

KARYOTYPES, BANDING PATTERNS AND NUCLEAR DNA CONTENT IN
CREPIDULA UNGUIFORMIS LAMARCK, 1822, AND *NATICARIUS*
STERCUSMUSCARUM (GMELIN, 1791) (MOLLUSCA, CAENOCASTROPODA)

Angelo Libertini¹, Roberto Vitturi², Armando Gregorini³ & Mariastella Colomba^{4*}

ABSTRACT

The chromosome complement and the nuclear DNA content in two caenogastropod species from the Mediterranean Sea, *Crepidula unguiformis* (Calyptraeidae) and *Naticarius stercusmuscarum* (Naticidae), were investigated by the application of both classical and molecular cytogenetic methods. Despite the constancy of haploid chromosome numbers ($n = 17$ in both species), *C. unguiformis* and *N. stercusmuscarum* show genome sizes amounting to 6.36 and 2.63 pg, respectively. Moreover, while *N. stercusmuscarum* resembles cytogenetically the other neotaenioglossan caenogastropods studied so far, *C. unguiformis* differs in: (i) number and location of rDNA clusters (ii), composition of telomeric repeats, and (iii) genome size, thus suggesting a peculiar karyotypical evolution undergone by this species.

Keywords: chromosomes, banding techniques, FISH, telomeres, genome size, Mollusca, Caenogastropoda.

INTRODUCTION

Cytogenetic information available in the literature on the molluscan superorder Caenogastropoda reports chromosome number, karyotypic macrostructure, chromosomal location of active nucleolus organizer regions (NORs) by silver staining, and/or genome size for approximately 130 species belonging to 20 families (Patterson, 1969; Thiriot-Quievreux, 2003; Gregory, 2008). Nearly nothing or very little is known about many caenogastropod families, including Calyptraeidae and Naticidae.

In the present paper we set out to investigate *Crepidula unguiformis* Lamarck, 1822 (Calyptraeidae), and *Naticarius stercusmuscarum* (Gmelin, 1791) (Naticidae) with the aim of contributing to fill the gap in cytogenetical knowledge of Caenogastropoda. For both species, the haploid chromosome number ($n = 17$) was the only datum available from the literature (Vitturi et al., 1982; Vitturi & Catalano, 1984). Chromosome analysis has been carried out using both classical (Giemsa-, silver- and fluorochrome staining, C-banding) and molecular [rDNA- and (TTAGGG)_n FISH] methods. Moreover, the genome size was determined for both species. Obtained results are compared to cytological data reported for other Caenogastropoda.

MATERIALS AND METHODS

Animals

Specimens of the two species were collected from the coast around Palermo (northwestern Sicily, Italy), Venice (northeastern Italy), and Fano (Marche Region, Central East Italy) in 2003, 2004, 2006 and 2007. *Crepidula unguiformis* is a protandric hermaphrodite, whereas *N. stercusmuscarum* is gonochoristic. Sexually mature individuals occurred from December to April (*C. unguiformis*) and from November to January (*N. stercusmuscarum*) and were collected only in Sicily. *Crepidula unguiformis* was typically found inside dead shells of *Astraea rugosa* (Linnaeus, 1758) and *Hexaplex trunculus* (Linnaeus, 1758).

Chromosome Preparations and Banding Methods

Spermatocytal and spermatogonial chromosome plates were obtained from testes of sexually mature individuals kept in 0.01% colchicine sea water for 2 hours. Gonads were dissected and treated following the air-drying technique described elsewhere (Vitturi, 1992).

Chromosomes were directly stained with Giemsa to describe their number and morphology,

¹Istituto di Scienze Marine, CNR, Riva 7 Martiri 1364/a, 30122 Venezia, Italy

²Dipartimento di Biologia Animale, Università di Palermo, via Archirafi 18, 90123 Palermo, Italy

³Istituto di Psicologia, Università di Urbino, via Ubaldini 17, 61029 Urbino (PU), Italy

⁴Istituto di Ecologia e Biologia Ambientale, Università di Urbino, via Maggetti 22, 61029 Urbino (PU), Italy

*Corresponding author: m.colomba@uniurb.it

or subjected to BSG C-banding (with barium hydroxide, saline and Giemsa, according to Sumner, 1990) to reveal constitutive heterochromatin, or banded with the fluorochromes DAPI (AT-specific) and CMA₃ (GC-specific) to show peculiar compartmentalisation of DNA (Sumner, 1990), or stained with silver nitrate to detect previously active nucleolar organizer regions (NORs) (Howell & Black, 1980).

Karyograms of *C. unguiformis* (eight spreads analysed) and *N. stercusmuscarum* (six spreads) were constructed from Giemsa-stained spermatogonial metaphases by pairing chromosomes according to dimensions and arm ratio. Nomenclature is according to Levan et al. (1964).

Fluorescent In Situ Hybridization (FISH)

FISH was performed on fixed chromosome plates from testis (as described by Vitturi et al., 2000) by hybridization with two different probes: the 18S rDNA partial sequence from sea urchin (*Paracentrotus lividus* Lamarck 1816) (Cantone et al., 1993) provided by R. Barbieri (University of Palermo) and (TTAGGG)_n vertebrate-type telomeric hexamer repeat. The latter probe was obtained by PCR in the absence of template (Ijdo et al., 1991) using (TTAGGG)₅ and (CCCTAA)₅ as primers. The PCR product was DIG-labelled following the random priming protocol (Roche). Nick translation labelling with digoxigenin of the 18S rDNA was performed following manufacturer's instructions (Roche). Slides were mounted in antifade solution containing propidium iodide (2 µg/ml) and observed on a Leica epifluorescence microscope equipped with a I3 filter set (BP 450–490, LP 515) allowing the simultaneous visualisation of both fluorescein-labelled hybrid (yellow) and chromosomal DNA (red).

Genome Size (GS) Evaluation

GS was evaluated through flow cytometric assay performed on cell suspensions of *C. unguiformis* (eight samples analysed from individuals collected in Venice) and *N. stercusmuscarum* (23 samples from individuals collected in Fano) obtained from deep frozen digestive gland. The analysis was comparatively extended to five specimens of the neogastropod *H. trunculus* (from Venice), the GS of which had already been determined by Pascoe et al. (2004) with a different method (Feulgen densitometry). Peripheral blood erythrocytes from chicken (2C GS = 2.5 pg, Tiersch et al., 1989) were added

to the molluscan cell suspension as internal standard. The methods for sample preparation and nuclear DNA content evaluation were the same described in Libertini et al. (2004). A BRITE-HS cytometer (Bio-Rad Laboratories) equipped with a xenon-mercury lamp was used for the analyses. Data were reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Chromosome Number and Karyotype

In both species the same diploid number $2n = 34$ was determined from counts of spermatogonial chromosomes, confirming the correspondent haploid value ($n = 17$) reported in previous papers (Vitturi et al., 1982; Vitturi & Catalano, 1984).

In the *C. unguiformis* karyotype (Fig. 1A) there are 11 metacentric/submetacentric and six subtelocentric (Fig. 1A – pairs 2, 5, 8, 13, 15, and 17) pairs. Chromosome length varies from 7.8 to 2.5 µm. In about one third of the observed metaphase figures, the chromosomes of pair 17 appear as heteromorphic. Such heteromorphism is likely due to different numbers of DNA units accumulated in the two homologues. In fact, the possibility that it may be linked to a sex determining mechanism is easily ruled out since hermaphrodite organisms cannot have sex linked chromosomes. Karyotype of *N. stercusmuscarum* (Fig. 1B) shows 12 metacentric/submetacentric and 5 subtelocentric/acrocentric (Fig. 1B – pairs 6, 11, 13, 16, and 17) homomorphic pairs. Chromosome length ranges from 3.5 to 1.2 µm.

BSG-C Banding, Fluorochrome Staining, rDNA FISH, and Ag-NOR Banding

BSG-C banding of spermatogonial chromosomes in *C. unguiformis* showed the occurrence of minute positive bands terminally and/or interstitially located (Fig. 2A), suggesting a low heterochromatin amount. In *N. stercusmuscarum*, dark heterochromatic blocks occupied the pericentromeric region of all chromosomes (Fig. 2B) plus the ends of the long arm in two large metacentric homologues which, according to their morphology and dimension, may be tentatively assigned to pair 3 of the karyotype (Fig. 2B, arrows; Fig. 1B).

In *C. unguiformis*, staining with DAPI did not reveal any brighter areas (Fig. 3A), whereas with CMA₃ (Fig. 4A) small positive interstitial

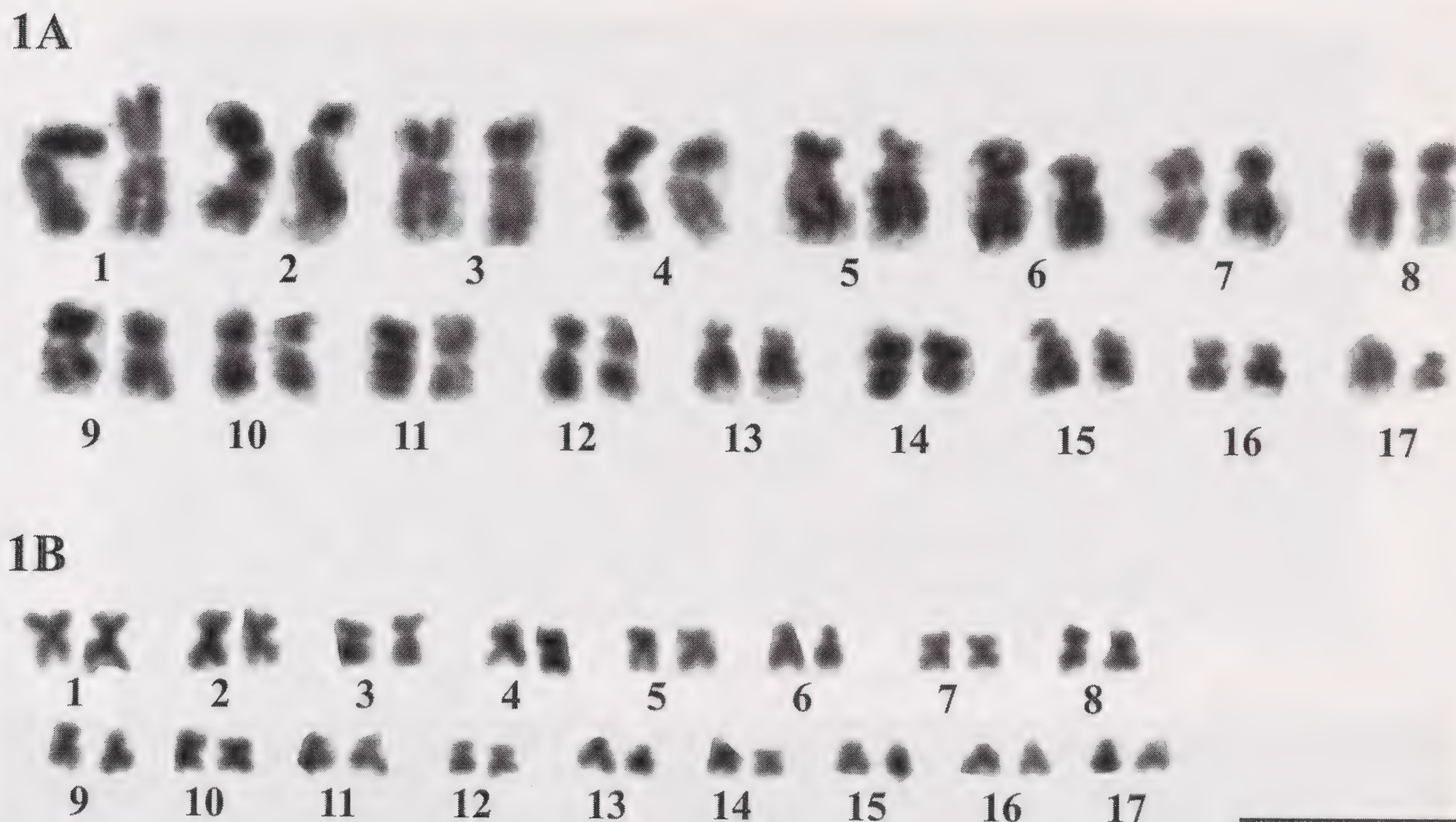


FIG. 1. Giemsa stained karyotypes: *C. unguiformis* (1A – pairs 2, 5, 8, 13, 15, and 17 are subtelocentric; the rest are metacentric/submetacentric,) and *N. stercusmuscarum* (1B – pairs 6, 11, 13, 16, and 17 are subtelocentric; the rest are metacentric/submetacentric). Scale bar = 10 μ m.

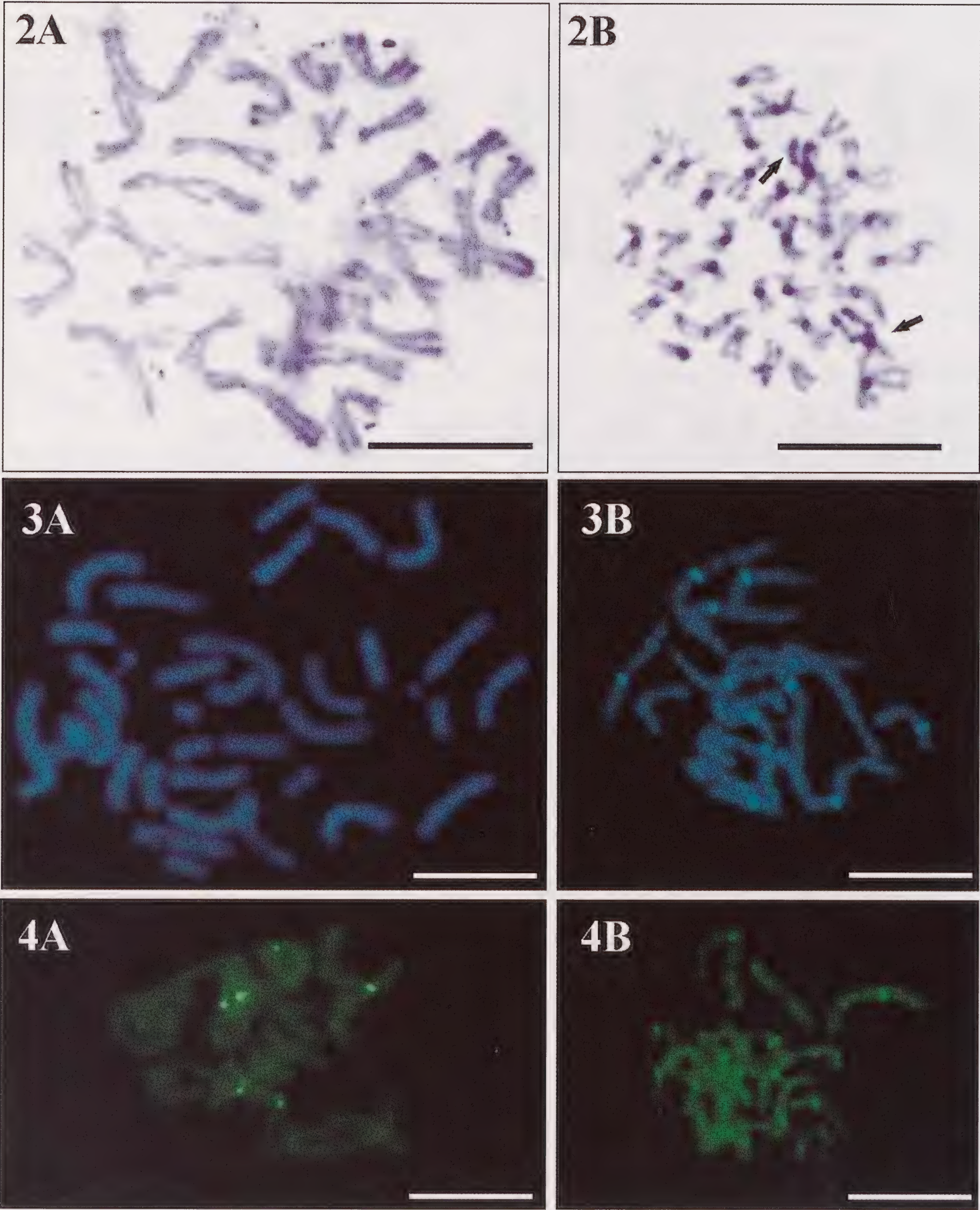
regions were visible on four early diakinetid bivalents. In *N. stercusmuscarum*, brighter spots could be observed in correspondence of heterochromatin after both DAPI (Fig. 3B) and CMA₃ (Fig. 4B) staining. Such a double positive fluorescence of heterochromatin seems unreliable, more probably depending on the high degree of DNA condensation rather than on the real DNA base-pair compartmentalisation.

In *C. unguiformis*, 18S rDNA FISH showed small interstitial hybridization signals near the centromeric region in eight spermatogonial chromosomes (14 metaphases analysed) (Fig. 5A) or in four early diakinetid bivalents (26 plates) (Fig. 6A). In bivalents, location of 18S rDNA is similar to CMA₃ brighter spots indicating that major ribosomal gene sites correspond to GC-rich DNA zones. In *N. stercusmuscarum*, major ribosomal sequences were mapped only on meiotic chromosomes and always appeared terminally located on one bivalent (42 plates) (Fig. 6B).

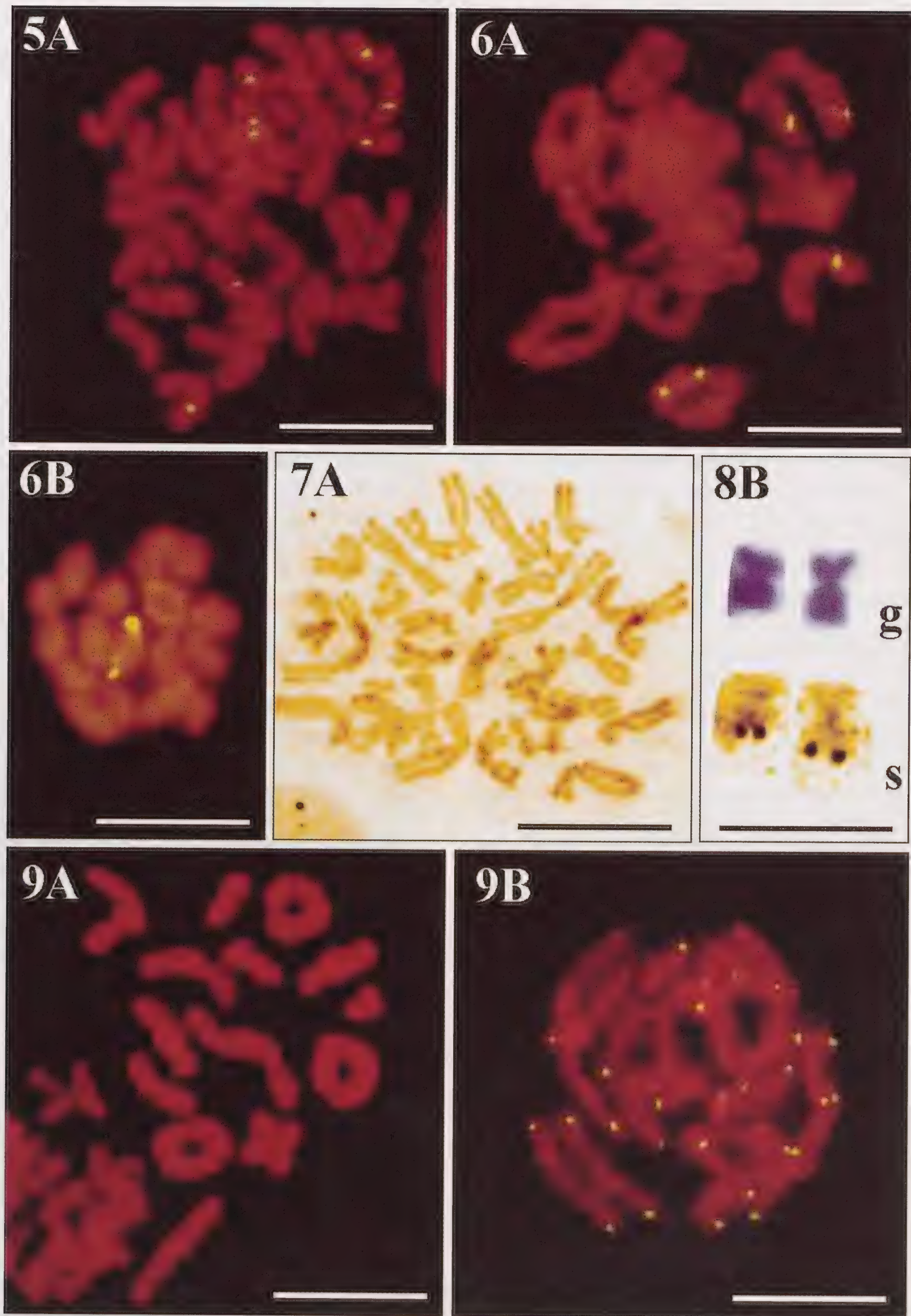
Ag-NOR banding did not provide repetitive argentophilic patterns in *C. unguiformis* (35 metaphases) (Fig. 7A). It is conceivable that the small-size of Ag spots, below the detection threshold of the silver impregnation technique, made difficult disclosure of 18S–28S rDNA clusters.

In 45 spermatogonial metaphases of *N. stercusmuscarum*, dark argentophilic spots were located in the terminal region of two large-sized elements (Fig. 8B), assigned to pair 3 of the karyotype (the same one interested by telomeric heterochromatin blocks).

In *N. stercusmuscarum*, major rDNA sites mapped on the terminal region of a single chromosome pair (two NORs), as already described for other species of Caenogastropoda (Colomba et al., 2002; Vitturi et al., 2002). Differently, in *C. unguiformis*, NORs were located in the interstitial region near the centromere of four chromosome pairs (eight NORs). If we accept the assumption that one pair of terminal NORs per cell is the plesiomorphic condition in both vertebrates (Schmid, 1978) and invertebrates, including molluscs (Thiriot-Quievreux & Insua, 1992; Pascoe et al., 1996), then, we are to conclude that an increase in NOR number has occurred in *C. unguiformis*, whereas the ancestral character (two NORs) has been conserved in *N. stercusmuscarum*. As concerns the mechanism responsible for such an increase, transposition of ribosomal cistrons either by non-homologous exchange or by NOR associated transposons could be the most probable rearrangement (Woznicki et al., 2000) responsible for the multiplication



FIGS. 2–4. C-banded spermatogonial metaphases of *C. unguiformis* (2A) and *N. stercusmuscarum* (2B), arrows indicate the NOR-bearing homologues (pair 3). DAPI stained plates: spermatogonial metaphase in *C. unguiformis* (3A) and spermatocytal pachytene in *N. stercusmuscarum* (3B). CMA₃ stained plates: early diakinetic bivalents in *C. unguiformis* (4A) and pachytene bivalents in *N. stercusmuscarum* (4B). Scale bars = 10 μ m.



FIGS. 5–9. *In situ* hybridization with 18S rDNA (from *Paracentrotus lividus*) to spermatogonial metaphase (5A) and early diakinetic bivalents (6A) in *C. unguiformis* and late diakinetic bivalents in *N. stercusmuscarum* (6B). Silver stained spermatogonial metaphase (7A) in *C. unguiformis* and the NOR-bearing chromosomes after Giemsa (g) and silver (s) staining in *N. stercusmuscarum* (8B). (TTAGGG)_n FISH: late diakinetic bivalents in *C. unguiformis* (9A) and pachytene bivalents in *N. stercusmuscarum* (9B). Scale bars = 10 μ m.

and dispersion of ribosomal regions. Nevertheless, the mechanism generating interstitial rDNA clusters from the ancestral terminal ones remains unclear. In our opinion, the intrachromosomal position may be hypothesized as the result of subsequent inversions involving NOR-bearing chromosomes.

(TTAGGG)_n FISH

In *C. unguiformis*, the telomeric (TTAGGG)_n probe did not label any chromosomal areas – neither terminal nor interstitial (Fig. 9A). Conversely, typical hybridized areas were clearly detectable at the ends of *N. stercusmuscarum* chromosomes (Fig. 9B). Since slides from both species were treated in the same FISH experiments (five replicates) and/or at the same experimental conditions, we rule out any technical bias underlying this finding.

The “vertebrate” telomere motif (TTAGGG)_n is considered the ancestral telomere repeat motif of Metazoa, conserved in most animal phylogenetic lineages with the up-to-date known exception of two major lineages: Arthropoda (with the TTAGG motif) and Nematoda (with TTAGGC/TTAGCA) (Sakai et al., 2005; Traut et al., 2007, and references therein). Nevertheless, in some species of molluscs, insects, spiders and annelids, telomeres do not hybridize with the expected telomeric probes and, at present, their sequences remain unidentified (Colomba et al., 2000; Vitturi et al., 2000; Frydrychova et al., 2004; Vitkova et al., 2005). (TTAGGG)_n is a widespread (i.e., Guo & Allen, 1997; Plohl et al., 2002; Colomba et al., 2002; Vitturi et al., 2005; Traut et al., 2007; present paper) but not the only motif of molluscan telomeres, since two species, the sacoglossan *Oxynoe olivacea* (Vitturi et al., 2000) and the caenogastropod *Crepidula unguiformis* (present paper) resulted as negative to FISH with this telomeric probe. Such a result might indicate that the telomeric motif (TTAGGG)_n was lost repeatedly and probably replaced with another repeat still unknown.

Genome Size

The mean C values of GS of *C. unguiformis*, *N. stercusmuscarum*, and *H. trunculus* were determined as 6.36 ± 0.11 pg, 2.63 ± 0.11 pg, and 2.31 ± 0.09 , respectively. *C. unguiformis* has more than twice the genome size of *N. stercusmuscarum*. Validity of the two data has been confirmed by the comparison with the C-value assessed herein for *H. trunculus* (2.31

pg), very close to 2.26 pg previously determined with Feulgen densitometry on gill cells of this species by Pascoe et al. (2004). The ratio between the GSs of *C. unguiformis* and *N. stercusmuscarum* is roughly corresponding to the ratio between their ranges of chromosome lengths (7.8–2.5 μ m vs. 3.5–1.2 μ m). In comparison with other Neotaenioglossan species (reference in Gregory, 2008), *C. unguiformis* and *N. stercusmuscarum* are endowed by the largest genome sizes. Although slightly larger, the value in *N. stercusmuscarum* resembles 2.40 pg assessed for another moon shell, *Polinices (Lunatia) heros* Say, 1822, by Hinegardner (1974). Conversely, *C. unguiformis* with 6.36 pg has one of the largest genome sizes up-to-now ascribed to any gastropod molluscs, only lower than four GS values found in the architaenioglossan Diplommatinidae, ranging from 6.70 to 8.04 pg (Ieyama & Ogaito, 2000).

Fluorochrome staining patterns showed a substantial homogeneity in base-pair (AT/GC) composition of nuclear DNA in the two species investigated. In particular, as also revealed by C-banding, *C. unguiformis* has a low amount of heterochromatin and all chromosomes show a dull staining with AT- or GC- fluorochrome, with the exception of NORs, being the only CMA₃ positive areas. Therefore, accumulation of repetitive DNA sequences (heterochromatin) may be excluded in *C. unguiformis* (unless repeated DNA escapes demonstration by standard methods here employed). For this reason, we suggest that euchromatic DNA may be involved in the increase of genome size observed in this species.

CONCLUSION

At a macroscopic scale, inter-specific karyological similarities (i.e., the same diploid number $2n = 34$ and the lack of any differentiated sex chromosome systems) can be found in the complements of *C. unguiformis* and *N. stercusmuscarum*. Nevertheless, the application of more powerful methods of investigation, as banding and *in situ* hybridization techniques, along with a comparison between present and compiled data on Caenogastropoda (reviewed in Thiriou-Quievreux, 2003; Gregory, 2008) reveal that *C. unguiformis* is cytogenetically differentiated in: (1) telomeric DNA composition, (2) rDNA clusters number and location, and (3) nuclear DNA content.

Although *C. unguiformis* and *N. stercusmuscarum* are phylogenetically distant they both

belong to the Hypsogastropoda, a suborder shown as monophyletic by several molecular analyses (Colgan et al., 2007, and reference therein). Moreover, within the Hypsogastropoda, by comparison of multi-gene sequences Colgan et al. (2007) differentiated the family Calyptraeidae from a set of families called as the "GC group". Present investigation also highlighted that the Calyptraeidae *C. unguiformis* underwent peculiar genomic changes by comparison with known cytogenetical data for the "GC group" families Littorinidae, Naticidae, Vermetidae, and Pterotracheidae (Vitturi et al., 1993, 1995, 1997; Thiriou-Quievreux & Seapy, 1997; Colomba et al., 2002; present paper). Nevertheless, future investigations on other Hypsogastropoda – with special reference to Calyptraeidae – will allow to understand if the cytogenetic diversity of *C. unguiformis* is limited to species- or genus-level or, otherwise, is shared with other species in the same family or suborder.

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